

In the matter of US Patent Application No. 10/535,433

I, Lorenzo Frigerio, currently residing at 1 Bromhurst Way, Warwick CV34 6NS, do hereby declare:

1. I am one of the inventors of US 10/535,433, a copy of which I have read and understood.

2. I am an expert in the field of the invention. My curriculum vitae is enclosed.

3. I have published over 30 papers. These are listed in the attached Annex.

4. I have read and understood the Official Action from the United States Patent and Trademark Office, dated 21 June 2007.

5. I declare that, prior to the present application, it was not known or expected that proteins originating from mammals, in particular humans, contain cryptic targeting signals which cause those mammalian, in particular human, proteins when expressed in a plant would cause the protein to be targeted to a particular location. I was surprised to find that proteins originating from mammals contain cryptic targeting signals which cause these mammalian proteins, when expressed in a plant, to be targeted to a particular location.

6. I declare that the constructs and methods of the present invention are novel and not obvious as compared to the methods and antibodies disclosed in Frigerio *et al* (Plant Physiology 123: 1483-1493 (August 2000) (hereinafter "Frigerio"), Vitale and Raikhel (Trends in Plant Science 4(4): 149-155 (April 1999)) (hereinafter "Vitale"), Koide *et al* (Plant Cell Physiol. (40)(11): 1152-1159 (1999)) (hereinafter "Koide"), Matsuoka and Neuhaus (J. Exp. Bot. 50: 165-174 (1999)) hereinafter ("Matsuoka").

7. I declare that Frigerio relates to the expression of immunoglobulin, *i.e.* a mammalian protein, in a plant. Frigerio identifies that the protein is delivered to two different sites in the plant: a portion of the protein is transported to the vacuole and a portion of the protein is secreted. However, Frigerio does not identify the underlying reason for the protein being delivered to two different sites of the plant. Frigerio does not identify a cryptic signal in the amino acid sequence of an immunoglobulin. Instead, Frigerio predicts that the reason might be:

(i) Due to the presence of thiol-mediated retention in the plant ER [endoplasmic reticulum], similar to thiol-mediated retention reported in mammalian cells (see page 1489, left hand column).

(ii) Due to the presence of conformational defects of tetramers or decamers that lead to their prolonged ER retention and eventual slow degradation (see page 1489, left hand column).

Furthermore, the Discussion of Frigerio states "*This raises the problem of which are the mechanisms and recognition events that lead to vacuolar delivery of a protein that is expected to be secreted*". The Discussion states "*To be sorted to the vacuole, soluble proteins need sorting signals; when these signals are deleted, the mutated proteins are secreted, albeit with variable efficiencies (Bednarek *et al.*, 1990, Crofts *et al.*, 1990. Although one potential receptor for vacuolar sorting has been identified, the mechanisms for vacuolar delivery are*

not yet fully clarified, and certainly more than one mechanism exists (Vitale and Raikhel, 1999). However, we were unable to demonstrate tight binding of IgA/G to endomembranes." This clearly shows that Frigerio had doubts about whether a dedicated targeting mechanism existed for IgA/G.

Thus it is clear that at the date of publication of Frigerio, the person skilled in the art did not know whether a targeting signal existed. In the unlikely event that the person skilled in the art suspected that a targeting signal existed, he would not know where to start looking for this targeting signal. Much further work was required to identify the targeting signal.

8. I declare that in order to identify whether a targeting signal exists I carried out the following investigations. It is important to note that I carried out the investigations in the order listed, that is, I believed that the first investigation was the most likely to show successful results and the last investigation was the least likely to show successful results:

- (i) Identification of whether saturation of the secretion machinery is responsible for a portion of the protein going to the vacuole.
- (ii) Identification of whether thiol-mediated retention (ie a form of quality control) is responsible for vacuolar targeting.
- (iii) Identification of whether a sequence in the constant alpha (Ca) domains of a hybrid IgA/G heavy chain is responsible for vacuolar targeting. I predicted that the Ca2 domain would be responsible for vacuolar targeting. Therefore, I carried out sequential deletions of the Ca domains in order to identify the targeting signal. I planned to delete the Ca domains in the following order: (1) Ca2, (2) Ca3.

My results showed (i) and (ii) not to be true. Only when a partial deletion of the C-terminus of Ca3 was attempted, as a last resort, we discovered that it lead to IgA/G secretion. Thus, what we believed was the least likely possibility proved in fact true.

9. I declare that as a person skilled in the art of the field of this invention. I would not combine the teachings of Frigerio with those of Vitale, Koide or Matsuoka. This is because Vitale, Koide and Matsuoka discuss the expression of plant proteins in a plant. In contrast, Frigerio discusses the expression of a mammalian protein in a plant. The person skilled in the art would not expect a mammalian protein to possess a targeting signal which can be recognised by the secretion machinery of a plant. Because vacuoles are not present in animal cells, and therefore the vacuolar sorting machinery is unique to plant cells, one would not expect animal proteins to carry intelligible vacuolar sorting signals.

Therefore, I was surprised to identify a targeting signal in a mammalian protein that is recognised by the secretion machinery of a plant.

10. In conclusion, I believe that Frigerio taken alone or in combination with Vitale, Koide or Matsuoka does not provide any motivation to generate the claimed invention. This is because it was surprising to discover that the underlying reason for heterologous mammalian protein being directed to the vacuole was due to the presence of a targeting signal. It was even more surprising that the targeting signal was located in the Ca domain of the protein. The degree of surprise is confirmed by the three years of research that was required to identify these facts.

I declare further that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine, or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of any patent issuing on this application.



Lorenzo Frigerio, Ph. D.

18 October 2007

Date

Curriculum Vitae

PERSONAL DETAILS

Full Name and Title: Dr Lorenzo Frigerio
Date of birth: November 9th, 1967
Department: Biological Sciences, University Of Warwick, UK

Education/Qualifications:

2001

Post-Graduate Certificate in Higher Education – ‘Warwick Teaching Certificate’
(approved by the Institute of Learning and Teaching)

1991-1994

Ph.D., Plant Genetics, Universita’ Cattolica S. Cuore, Piacenza, Italy
Thesis: Expression of bacterial genes for enzymes of the aspartate biosynthetic pathway in transgenic tobacco.

1986 - 1991

Degree in Agricultural Sciences (grade: 110/110 *cum laude*), Universita’ Cattolica S. Cuore, Piacenza, Italy. Thesis: isolation of promoter sequences in plants via random *GUS* fusions.

Appointments held:

2005-Present

Senior Lecturer, Department of Biological Sciences, University of Warwick

1999-2005

Lecturer, Department of Biological Sciences, University of Warwick.

1998 - 1999

BBSRC - funded Postdoctoral Research Fellow at the Department of Biological Sciences, University of Warwick, Coventry. Supervisor: Dr L. M. Roberts. Project: “Endoplasmic reticulum associated protein degradation in plants”.

1997- 1998

Postdoctoral Research Fellow at the Italian National Research Council (CNR), at the Istituto Biosintesi Vegetali CNR, Milano, Italy. Supervisor: Dr A. Vitale. Project: “Intracellular transport of phaseolin and interactions with components of the plant secretory pathway”.

1995 - 1997

EU funded Postdoctoral Research Fellow at the Department of Biological Sciences, University of Warwick, Coventry (UK). Supervisor: Dr. L. M. Roberts. Project:

"Analysis of the functional components and mechanisms controlling the plant endomembrane system".

1994- 1995

Graduate Research Assistant, Istituto di Genetica, Università Cattolica S. Cuore, Piacenza (Italy), November 23-August 5. Prof. C. Lorenzoni. "Expression and secretion of a fungal phytase gene in yeast".

1994

Visiting scientist , MSU-DOE Plant Research Laboratory, Michigan State University (USA), July 1 - November 21. Prof. N.V. Raikhel. Project: "Expression and regulation of the *Arabidopsis thaliana* *aERD2* gene".

1991

Placement at the Department of Plant Biology, Scripps Research Foundation, La Jolla, California (USA), September 18 - November 20. Dr. A. C. Hiatt. Project: "Expression of monoclonal antibodies in transgenic plants".

Publications and patents:

Note:

First authorship indicates that I performed the vast majority of the experimental work presented.

Last (corresponding) authorship indicates that I conceived the project, planned and supervised the experimental work, and wrote the article.

Where I am not first or last author, I provide an estimate of my contribution to the work. This is expressed as a percentage of the experimental data (i.e. figures) presented in the article.

Research Articles (refereed journals):

Manuscripts currently submitted for publication:

Paul MJ, Craddock CP, Jolliffe NA, Nuttall J, **Frigerio L**. The hub of the *Arabidopsis* clathrin heavy chain interacts with endogenous clathrin chains but does not affect anterograde protein transport.

Marshall RS, Jolliffe NA, Ceriotti A, Lord JM, **Frigerio L**, Roberts LM. Characterisation of ER to cytosol retro-translocation in plant cells.

Accepted manuscripts

Tolley N, Sparkes IA, Hunter PR, Craddock CP, Nuttall J, Roberts LM, Hawes C, Pedrazzini E, **Frigerio L**. Overexpression of a plant reticulon remodels the lumen of the cortical endoplasmic reticulum but does not perturb protein transport. *Traffic*, accepted pending minor revisions, 10/09/07 (currently resubmitted)

Articles in print:

1. Hunter PR, Craddock CP, Di Benedetto S, Roberts LM, **Frigerio L**. (2007) Fluorescent reporter proteins for the tonoplast and the vacuolar lumen identify a single vacuolar compartment in *Arabidopsis* cells. *Plant Physiol*, published on September 28, 2007; DOI10.1104/pp.107.103945
2. Marusic C, Nuttall J, Buriani G, Lico C, Baschieri S, Ma J-KC, Benvenuto E, **Frigerio L** (2007). Expression and intracellular targeting of HIV nef variants in transiently transformed and transgenic tobacco cells. *BMC Biotechnol*. 7:12
3. Maggio C, Barbante A, Ferro F, **Frigerio L**, Pedrazzini E (2007) Intracellular sorting of the tail-anchored protein cytochrome b5 in plants: a comparative study

using different isoforms from rabbit and Arabidopsis. *J. Exp. Bot.* 58:1365-79 (my contribution: 40% - all confocal microscopy)

4. Jolliffe NA, Di Cola A, Marsden CJ, Lord JM, Ceriotti A, **Frigerio L**, Roberts LM (2006). The N-terminal ricin propeptide influences the fate of ricin A-chain in tobacco protoplasts. *J. Biol. Chem.* 281: 23377-23385 (my contribution: 10%)
5. Obregon P, Chargelegue D, Drake PMW, Prada A, Nuttall J, **Frigerio L**, Ma J-KC (2006). HIV-1 p24-Immunoglobulin fusion molecule: A new strategy for plant-based protein production. *Plant Biotechnol. J.* 4:195-207 (my contribution: 20%)
6. Nuttall, J, Ma, JK-C, **Frigerio, L** (2005). A functional antibody lacking N-linked glycans is efficiently folded, assembled and secreted by tobacco mesophyll protoplasts. *Plant Biotechnol. J.* 3:497-504
7. Di Cola A, **Frigerio L**, Lord JM, Roberts LM, Ceriotti, A (2005). Endoplasmic reticulum-associated degradation of ricin A chain has unique and plant-specific features. *Plant Physiol.* 137: 287-296. (my contribution: 20%)
8. Jolliffe NA, Brown JC, Neumann U, Vicre' M, Bachi A, Hawes C, Ceriotti A, Roberts LM*, **Frigerio L*** (2004). Transport of ricin and 2S albumin precursors to the storage vacuoles of *Ricinus communis* endosperm involves the Golgi and VSR-like receptors. *Plant J.* 39:821-833 (*joint corresponding authors)
9. Brown JC, Jolliffe NA, **Frigerio, L**, Roberts, LM (2003). Sequence-specific, Golgi-dependent targeting of the castor bean 2S albumin to the vacuole in tobacco protoplasts. *Plant J.* 36:711-719 (my contribution: 20%)
10. Foresti O*, **Frigerio L***, Holkeri H, de Virgilio M, Vavassori S, Vitale A (2003). A phaseolin domain directly involved in trimer assembly is a BiP binding determinant. *Plant Cell* 15:2464-2475 (* joint first authors)
11. Hadlington, J, Santoro, A, Nuttall, J, Denecke, J, Ma, J, Vitale A, **Frigerio L** (2003). The C-terminal extension of a hybrid immunoglobulin A/G heavy chain is responsible for its Golgi-mediated sorting to the vacuole. *Mol. Biol. Cell* 14:2592-2602.
12. Nuttall, J, Vitale, A, **Frigerio, L** (2003). C-terminal extension of phaseolin with a short methionine-rich sequence can inhibit trimerisation and result in high instability. *Plant Mol. Biol.* 51:881-890
13. Jolliffe, N. A., Ceriotti, A., **Frigerio, L.** and Roberts, L. M. (2003). The position of the proricin vacuolar targeting signal is functionally important. *Plant Mol. Biol* 51:631-641 (my contribution: 25%)

14. Nuttall, J., Vine, N., Hadlington, J., Drake, P., **Frigerio, L.***, Ma, J K-M* (2002). ER-resident chaperone interactions with recombinant antibodies in transgenic plants. *Eur. J. Biochem.* 269:6042-6051. (* joint corresponding authors)
15. Di Cola A, **Frigerio L**, Lord JM, Ceriotti A, Roberts LM (2001) Ricin A chain without its partner B chain is degraded after retrotranslocation from the endoplasmic reticulum to the cytosol in plant cells. *Proc Natl Acad Sci USA* 98:14726-14731. (my contribution: 30%)
16. **Frigerio, L.***, Jolliffe, N. A., Di Cola, A., Hernández Felipe, D., Paris, N., Neuhaus, J.-M., Lord, J. M. and Roberts, L. M. (2001). The internal propeptide of the ricin precursor carries a sequence-specific determinant for vacuolar sorting. *Plant Physiol* 126: 167-175 (* corresponding author as well as first author)
17. **Frigerio, L.**, Pastres A, Prada A, Vitale A (2001). Influence of KDEL on the fate of trimeric or assembly-defective phaseolin: selective use of an alternative route to vacuoles. *Plant Cell* 13: 1109-1126
18. **Frigerio, L***, Foresti, O., Hernandez-Felipe, D., Neuhaus, J.-M., Vitale, A. (2001). The C-terminal tetrapeptide of phaseolin is sufficient to target green fluorescent protein to the vacuole. *J. Plant Physiol* 158: 499-503 (* corresponding author as well as first author)
19. **Frigerio, L**, Vine, ND, Pedrazzini, E, Hein, MB, Wang, F, Ma, J K-C, Vitale, A (2000). Assembly, secretion and vacuolar delivery of a hybrid immunoglobulin in plants. *Plant Physiol.* 123: 1483-1493
20. **Frigerio, L.**, de Virgilio, M., Prada, A., Faoro, F., Vitale, A. (1998). Sorting of phaseolin to the vacuole is saturable and requires a short C-terminal peptide. *Plant Cell* 10: 1031-1042.
21. **Frigerio, L.**, Vitale, A., Lord, J.M., Ceriotti, A., Roberts, L.M. (1998). Free ricin A chain, proricin and mature toxin have different cellular fates when expressed in tobacco protoplasts. *J. Biol. Chem.* 273: 14194-14199.
22. Pedrazzini, E., Giovinazzo, G., Bielli, A., de Virgilio, M., **Frigerio, L.**, Pesca, M., Faoro, F., Bollini, R., Ceriotti, A., Vitale, A. (1997). Protein quality control along the route to the plant vacuole. *Plant Cell* 9, 1869-1880. (my contribution: 10%)
23. Bar-Peled, M., da Silva Conceição, A., **Frigerio, L.**, Raikhel, N.V. (1995). Expression and regulation of aERD2, a gene encoding the KDEL receptor homologue in plants, and other genes encoding proteins involved in ER -Golgi vesicular trafficking. *Plant Cell* 7: 667-676. (my contribution: 30%)
24. Fogher, C., **Frigerio, L.**, Delledonne, M. (1995). Inoculation of genetically modified strains of Azospirillum: monitoring of population dynamics. In: Fendrik, I. (ed.), NATO ASI Series vol. G, Azospirillum VI and related microorganisms:

Genetics, Physiology. Springer-Verlag, Heidelberg, 37: 515:521 (my contribution: 30%)

25. **Frigerio, L.**, Delledonne, M., Chiusa, G., Fogher, C. (1993). Polymorphism of the phytase gene in the genus *Aspergillus*. *Mol. Biol. (Life Sci Adv.)* 12: 191-196.

Refereed reviews::

1. Paul, MJ, **Frigerio, L** (2007). Coated vesicles in plant cells. *Sem. Cell Dev. Biol.*, 18: 471-478
2. Jolliffe, NJ, Craddock, CP, **Frigerio, L** (2005). Pathways for protein transport to seed storage vacuoles. *Biochem. Soc. Trans.* 33:1016-1018
3. **Frigerio, L.** (2002). Visualising plant cell biology. *Trends Plant Sci.* 7:423
4. Lord, J.M., **Frigerio, L.** (2002). ER quality control: a function for sugars in the cytosol. *Curr. Biol.* 12: R663-R665
5. **Frigerio, L.**, Lord, J.M. (2000). Glycoprotein degradation: do sugars hold the key? *Curr Biol.* 10: R674-R677
6. Lord, J.M., Davey, J., **Frigerio, L.**, Roberts, L.M. (2000). Endoplasmic Reticulum-associated protein degradation. *Sem. Cell Dev. Biol.* 11: 159-164
7. **Frigerio, L.**, Roberts, L.M. (1998). The enemy within: ricin and plant cells. *J. Exp. Bot.* 49, 1473-1480
8. **Frigerio L.**, Pedrazzini, E., Giovinazzo, G., Bollini,R., Ceriotti, A. Vitale, A. (1996). The synthesis of phaseolin: a model for the study of the plant secretory pathway. *Giornale Botanico Italiano* 130, 891-900.

Book Chapters:

1. Drake, PMW, Chargelegue, DM, Obregon, P, Prada, A, **Frigerio, L**, Ma, J (2003). The assembly and potential applications of immunoglobulins expressed in transgenic plants in Plant Biotechnology 2002 and beyond, I Vasil (ed.), Kluwer Academic, pp 363-370.
2. Ceriotti, A., Paris, N., Hillmer, S., **Frigerio, L.**, Neuhaus, J.-M., Vitale, A., Robinson, D.G. (2003). Plant Cell Biology. In *Methods in Cell Biology* (Oxford University Press), pp133-161

Patent:

International patent n. WO2004046190 – Hadlington, J and **Frigerio, L.** Antibodies lacking vacuolar targeting signal peptide and capable of binding J-chain. Publication date 03/06/2004.